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Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

**R C van Dijk**

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Novel CC-chemokine-binding tick proteins

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## NOVEL CC-CHEMOKINE-BINDING TICK PROTEINS

### FIELD OF THE INVENTION

This patent describes novel CC-chemokine binding proteins.

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### BACKGROUND OF THE INVENTION

Chemokines are small, secreted pro-inflammatory proteins, which mediate directional migration of leukocytes from the blood to the site of injury. Depending on the position of the conserved cysteines characterizing this family of proteins, the chemokine family can be divided structurally in C, C-C, C-X-C and C-X<sub>3</sub>-C chemokines, to which corresponds a series of membrane receptors (Baggiolini M et al., 1997; Fernandez EJ and Lolis E, 2002).

A series of membrane receptors, all heptahelical G-protein coupled receptors, are the binding partners that allow chemokines to exert their biological activity on the target cells, which may present specific combinations of receptors according to their state and/or type. The physiological effects of chemokines result from a complex and integrated system of concurrent interactions: the receptors often have overlapping ligand specificity, so that a single receptor can bind different chemokines, as well a single chemokine can bind different receptors.

Studies on structure-activity relationships indicate that chemokines have two main sites of interaction with their receptors, the flexible amino-terminal region and the conformationally rigid loop that follows the second cysteine. Chemokines are thought to dock onto receptors by means of the loop region, and this contact is believed to facilitate the binding of the amino-terminal region that results in receptor activation.

Usually chemokines are produced at the site of injury and cause leukocyte migration and activation, playing a fundamental role in inflammatory, immune,

homeostatic and angiogenic processes. These molecules, therefore, are considered good target candidates for therapeutic intervention in diseases associated to such processes, by inhibiting specific chemokines and their receptors at the scope to modulate leukocytes maturation, recruitment, and activation, as well as other physiological activities such as angiogenesis or arteriosclerosis (Baggiolini M, 2001; 5 Loetscher P and Clark-Lewis I, 2001; Godessart N and Kunkel SL, 2001).

In addition to mutant inhibitory chemokines, antibodies, and inhibitors blocking the receptors (small molecules), the search for effective chemokine antagonists has been extended also to a series of virus and other organisms that, when entering in 10 contact with humans or mammals, showed potent immunomodulatory activities affecting the animal.

The viral mimicry of cytokines, chemokines, and their receptors has been may indicate strategies of immune modulation for developing therapeutic products (Alcami A, 2003; Lindow M et al., 2003). Recently, immunomodulatory factors of 15 haematophagous arthropods (such as mosquitoes, sandflies and ticks) have been reviewed (Gillespie, RD et al., 2000; Nuttall PA et al., 2000; Schoeler GB and Wikel SK, 2001).

In particular, the salivary glands of ticks produce a complex mixture of bioactive molecules having anti-inflammatory, anti-haemostatic and anti-immune molecules. 20 These include bioactive proteins that control histamine, bind immunoglobulins, inhibit the alternative complement cascade or other proteases of distinct classes, and several peptide families of unknown function displaying different conserved cysteine residues, many of which contain single Kunitz domains.

The effect of these molecules is, probably, to provide a privileged site at the tick-host interface that shelters tick from the normal innate and acquired host immune mechanisms that combat infections, ensuring successful feeding.

Moreover, tick salivary glands are considered the major route by which tick-borne pathogens enter the host during feeding, since ticks use their salivary glands as a means of concentrating the blood meal by returning the excess fluid and ions back to the host, possibly transmitting pathogens hosted in these glands. In fact, tick induced modulation of host immunity is increasingly recognized as an important factor in successful transmission or establishment of tick-borne pathogens.

Many other immunomodulating activities have characterized in tick saliva extracts, or for specific tick sequences (Alarcon-Chaidez FJ et al., 2003; Bergman DK et al., 2000; Anguita J et al., 2002; Gwakisa P et al., 2001; Leboulle G et al., 2002; Kopecky J et al., 1999; Kovar L et al., 2002; Gillespie RD et al., 2001).

At the level of protein extract, the saliva from *Rhipicephalus sanguineus* inhibits antigen-stimulated production of immunoglobulins and IFN-gamma, IL-2 and IL-5 expression in a dose-dependent manner (Matsumoto K et al., 2003; Matsumoto, K et al., 2001).

CXC-chemokine binding activities, in particular CXCL8 / Interleukin 8 binding activities, have been detected (but not characterized in terms of specific protein sequences) in the saliva prepared from several ixodid tick species (*Dermacentor reticulatus*, *Amblyomma variegatum*, *Rhipicephalus appendiculatus*, *Haemaphysalis inermis*, *Ixodes ricinus*), demonstrating a reduction of the level of detectable IL-8, and inhibiting IL-8 induced chemotaxis of human blood granulocytes. (Hajnicka V et al., 2001; Kocakova P et al. 2003).



Antigens from *Rhipicephalus sanguineus* ticks elicit potent cell-mediated immune responses in resistant but not in susceptible animals. The saliva introduced during tick infestations reduces the ability of a susceptible animal host to respond to tick antigens that could stimulate a protective immune response. As a consequence, the animals present a lack of DTH response and disturbed cellular migration to tick feeding site, which can represent a deficient response against ticks (Ferreira BR et al., 2003).

A homologue of the pro-inflammatory cytokine Macrophage Migration Inhibitory Factor in the tick *Amblyomma americanum*. This sequence, in an in vitro functional assay, inhibited the migration of human macrophages to the same extent that recombinant human MIF did (Jaworski DC et al., 2001).

Despite the large amount of literature, only a few articles list cDNA sequences identified by random cDNA sequencing and differential screens of libraries generated from various tick tissues and/or species that have been extensively characterized. Lists of cDNA sequences have been published for two developmental stages of *Amblyomma americanum* and of *Dermacentor andersoni* (Hill CA and Gutierrez JA, 2000), salivary glands of unfed and fed male *Amblyomma americanum* (Bior AD et al., 2002), male mating *Ixodes scapularis* (Packila M and Guilfoile PG, 2002), salivary glands of *Amblyomma variegatum* (Nene V et al., 2002) and of *Ixodes scapularis* (Valenzuela JG et al., 2002; Francischetti IM et al., 2002).

However, most of these sequences are not characterized biochemically, and many annotations are entered only the basis of sequence similarity with known proteins involved in basic cellular functions, such as previously characterised in tick salivary glands for enzyme activities or inducing antibody response.

In particular, there is not indication in the prior art that a specific protein isolated from a tick may act as a CC-chemokine binding protein and a CC-chemokine antagonist.

5

## SUMMARY OF THE INVENTION

It has been surprisingly found that saliva of *Rhipicephalus sanguineus* (dog tick) contains CC-chemokine binding activities. In particular, rsChBP-I, a novel protein isolated from a *Rhipicephalus sanguineus* cDNA library and expressed in mammalian cells, competes with a CC-chemokine receptor for CC-chemokine binding. This protein  
10 belongs to a new class of tick proteins that can be used therapeutically as antagonists of natural CC-chemokines in mammalian organisms, as well as targets for vaccination and for the control of ticks and of tick-borne pathogens.

Other features and advantages of the invention will become evident from the following detailed description.

15

## DESCRIPTION OF THE FIGURES

Figure 1: binding of radiolabelled chemokines to *Rhipicephalus sanguineus* saliva extracts (rsSE) or a CC-chemokine-binding protein from ectromelia virus (vCCI). The extract and the protein were spotted in parallel onto different nitrocellulose filters in the indicated amount (top left cell), then each filter  
20 was incubated with the specific radiolabeled chemokine indicated in the column (right).

Figure 2: biochemical characterization of CC-chemokine-binding activities in the saliva of *Rhipicephalus sanguineus* using a Scintillation Proximity Assay (SPA). The interaction between radiolabelled CCL / MIP-1alpha and CCR1  
25

immobilized on SPA beads was measured without a competitor, with the natural competitor (MIP-1alpha), or with two amounts of tick saliva protein extract. A similar profile was obtained using the same SPA beads and radiolabeled or unlabeled CCL5 / RANTES.

5     Figure 3 : detection of CC-chemokine-binding activity in HEK293 culture medium by chemical cross-linking to <sup>125</sup>I-MIP-1alpha. (A) Titration of the positive control (the viral CC-chemokine binding protein vCCI) added to HEK293 culture medium in the indicated amount and in presence of the cross-linking agent (BS<sup>3</sup>). The free radiolabeled CC-chemokine migrates as a 8 kDa band. The  
10     radiolabeled cross-linked complex formed by the CC-chemokine and vCCI migrate as a 35-45 kDa band. (B) Screening of individual clones from the *Rhipicephalus sanguineus* cDNA expression library expressed in HEK293 mammalian cells. The signal observed in the cross-linking experiment with the culture medium of a specific HEK293 clone transformed with this cDNA  
15     library (Clone2) is compared the signal obtained with HEK293 culture medium containing vCCI, in presence (lanes +) or in absence (lanes -) of the cross-linking reagent (BS3) The free radiolabeled CC-chemokine and the cross-linked complexes (between the radiolabeled CC-chemokine and the tick or viral CC-chemokine binding protein) are indicated.

20     Figure 4: Clone2 DNA sequence (SEQ ID NO: 3), including the ORF encoding for the amino acid sequences of rsChBP-I (SEQ ID NO: 4) The coding portion of the DNA is aligned with the amino acid sequence. The signal sequence (predicted by the algorithm SIGNALJ) is underlined. The predicted polyadenylation sites are boxed.

Figure 5: alignment of the amino acid sequences of rsChBP-I (SEQ ID NO: 4) with avChBP-I (SEQ ID NO: 6) and isChBP-I (SEQ ID NO: 8), two protein sequences encoded by ORFs identified in non-annotated *Amblyomma variegatum* and *Ixodes scapularis* cDNAs, respectively. The numbering corresponds to the nucleotide position in the respective cDNA sequences Clone2 (SEQ ID NO: 3), BM289643 (SEQ ID NO: 5), and AF483738 (SEQ ID NO: 7). Identical and conserved (indicated with +) residues between rsChBP-I and avChBP-I, and between rsChBP-I and isChBP-I are indicated in bold.

Figure 6: CC-chemokine binding activity of recombinant rsChBP-I expressed in HEK293 culture medium. The interaction between radiolabeled CCL3 / MIP-1alpha and CCR1 is measured in a Scintillation Proximity Assay performed with or without the natural competitor, or with an increasing amount of culture medium from HEK293 cells expressing rsChBP-I added to the sample.

#### DETAILED DESCRIPTION OF THE INVENTION

In view of the above mentioned evidences in the prior art, there is no indication that specific binding CC-chemokine binding proteins are present in tick saliva, neither as activities detectable in the saliva, nor as protein sequences homologous to any known CC-chemokine binding protein characterized in other organisms.

The present invention provides novel tick protein sequence having CC-chemokine binding properties characterized by the means of activity-based screening of tick saliva extracts coupled to a cDNA library screening, allowing the identification of a novel family of tick immunomodulatory proteins.

The main objects of the present invention are proteins having CC-chemokine binding activity isolated from a tick belonging to the *Ixodida* suborder. These proteins can be expressed in the salivary gland and/or isolated from the saliva of these organisms.

5       The proteins of the invention can be expressed from ticks belonging to different ticks families, in particular the ones belonging to the *Ixodidae* family. The examples disclose protein sequences isolated in tick belongs to the *Rhipicephalinae*, *Amblyomminae*, and *Ixodinae* subfamilies, and more in particular from *Rhipicephalus sanguineus*, *Amblyomma variegatum*, and *Ixodes scapularis*.

10       A further object of the invention are protein having a polypeptide sequence at least 80% homologous to any of the specific polypeptide sequences disclosed for the tick species above indicated: rsChBP-I (SEQ ID NO: 4), avChBP-I (SEQ ID NO: 6), and isChBP-I (SEQ ID NO: 8). Moreover, the homology amongst these three sequences (Figure 6) allow to identify a family of novel CC-chemokine binding proteins that can be  
15 eventually identified and isolated from an organism not belonging to the *Ixodida* suborder. These proteins should have a polypeptide sequence at least 80% homologous to rsChBP-I (SEQ ID NO: 4) and a Cysteine residue in the positions corresponding to residues 40, 59, 64, 76, 86, 98, and 99 in rsChBP -I.

20       The examples show that the protein of the invention bind CC-chemokines and compete with the specific CC-chemokine receptor, in particular when the CC-chemokine binds the chemokine receptor CCR1, such as the CCL3 / MIP-1alpha. However, it cannot be excluded that the protein s of the invention can bind to CC-chemokine that is a non-mammalian CC-chemokine analogue expressed by the tick itself, or by any virus or bacteria hosted by the tick.

The proteins of the invention can be in a mature form resulting from one or more post-translational modifications (glycosilation, phosphorylation, modification with endo-/exopeptidase for eliminating the signal peptide, for example). The presence and the extension of signal peptides not essential for CC-chemokine binding can be easily  
5 predicted using one of the available algorithm (Figure 4).

These proteins, whenever the encoding DNA sequence has been cloned and characterized, can be also in a recombinant form expressed by a prokaryotic or eukaryotic host cells wherein said sequence has been appropriately introduced. The examples show the expression of one of these sequences in a mammalian cell line.

10 A further object of the invention are active mutants, variants, or functional equivalents of a CC-chemokine binding proteins identified in terms of homology to the disclosed sequences of rsChBP -I (SEQ ID NO: 4), avChBP-I (SEQ ID NO: 6), and isChBP-I (SEQ ID NO: 8). These alternative compounds, wherein one or more amino acid residues can be added, deleted, or substituted, are defined as molecules with  
15 changes to the sequences above indicated that do not affect the basic characteristics disclosed in the present invention (i.e. CC -chemokine binding activity). The CC-chemokine binding properties of the active mutants, variants, or functional equivalents should be at least comparable, or even higher, as determined by means known in the art and disclosed in the Examples below.

20 Similar active mutants can be natural, as the ones corresponding to a paralog identified in another organism, such a different tick species belonging to the same family. But, in most cases, these sequences are synthetic or artificial, which can be prepared by known chemical synthesis, recombinant DNA technology, site -directed mutagenesis, or any other known technique suitable thereof, which provide a finite set  
25 of substantially corresponding mutated or shortened peptides or polypeptides which

can be routinely obtained and tested by one of ordinary skill in the art using the teachings presented in the prior art and in the Examples of the present patent application.

The term "active" means that such compounds should maintain the functional features of the CC-chemokine proteins of the present invention, and should be as well acceptable for any of the claimed uses and methods.

Preferred changes in these active mutants are commonly known as "conservative" or "safe" substitutions, and involve non-basic residues. Conservative amino acid substitutions are those with amino acids having sufficiently similar chemical properties, in order to preserve the structure and the biological function of the molecule. It is clear that insertions and deletions of amino acids may also be made in the above defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, e.g., under ten, and preferably under three, and do not remove or displace amino acids which are critical to the functional conformation of a protein or a peptide.

The literature provide many models on which the selection of conservative amino acids substitutions can be performed on the basis of statistical and physico-chemical studies on the sequence and/or the structure of natural protein (Rogov SI and Nekrasov AN, 2001). Protein design experiments have shown that the use of specific subsets of amino acids can produce foldable and active proteins, helping in the classification of amino acid "synonymous" substitutions which can be more easily accommodated in protein structure, and which can be used to detect functional and structural homologs and paralogs (Murphy LR et al., 2000). The synonymous amino acid groups and more preferred synonymous groups are those defined in Table I.

A common example of such alternative sequences are fusion proteins comprising a part or an entire CC-chemokine binding protein of the invention and an amino acid sequence belonging to a protein sequence other than the corresponding CC-chemokine binding protein, such as an amino acid sequence belonging to one or more  
5 of these protein sequences: extracellular domains of membrane-bound protein, immunoglobulin constant region, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins.

This heterologous sequence in the fusion protein should provide additional properties without impairing significantly the applicability. Examples of such additional  
10 properties are an easier purification procedure, a longer lasting half-life in body fluids, or extracellular localization. This latter feature is of particular importance for defining a specific group of fusion or chimeric proteins included in the above definition since it allows these monomeric variants to be localized in the space where not only where the isolation and purification of these peptides is facilitated, but also where CC-chemokines  
15 naturally interact with other molecules.

Alternatively, the active mutant may result from sequence alterations reducing the immunogenicity of said CC-chemokine binding protein when administered to a mammal. The literature provides many example on these sequence alterations can be designed and introduced at this scope or for other functional optimizations that allow a  
20 safe and effective administration of a therapeutic protein, especially when it is non-human, non-mammalian, or non-natural protein (Vasserot AP et al., 2003; Marshall SA et al., 2003; Schellekens H, 2002; Gendel SM, 2002; WO 03/104263; WO 03/006047; WO 02/98454; WO 02/96454; WO 02/79415; WO 02/79232; WO 02/66514; WO0140281; WO 98/52976; WO 96/40792; WO 94/11028).



A further object of the invention are fragments of a CC-chemokine binding protein  
a CC-chemokine binding proteins identified in terms of homology to the disclosed  
sequences of rsChBP-I (SEQ ID NO: 4), avChBP-I (SEQ ID NO: 6), and isChBP-I  
(SEQ ID NO: 8), having an immunizing activity when administered to a mammal. These  
5 fragments should have appropriate antigenic, immunogenic properties for raising a  
immunitary response when needed (for example, against ticks or tick-borne pathogenic  
organisms). The literature provides many examples on how such functional sequences  
can be identified as candidate vaccine antigens, and eventually administered with  
adjuvants and/or cross-linked to a carrier (Mulenga A et al. 2000; WO 01/80881; WO  
10 03/030931; WO 01/87270; WO 95/22603).

The proteins of the present invention can be produced, formulated, administered,  
or generically used in the form of their active fractions, precursors, salts, or derivatives.

The term "fraction" refers to any fragment of the polypeptidic chain of the  
compound itself, alone or in combination with related molecules or residues bound to it,  
15 for example residues of sugars or phosphates, or aggregates of the original  
polypeptide or peptide. Such molecules can result also from other modifications which  
do not normally alter primary sequence, for example *in vivo* or *in vitro* chemical  
derivativization of peptides (acetylation or carboxylation), those made by modifying the  
pattern of phosphorylation (introduction of phosphotyrosine, phosphoserine, or  
20 phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes  
which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes)  
of a peptide during its synthesis and processing or in further processing steps.

The "precursors" are compounds which can be converted into the compounds of  
present invention by metabolic and enzymatic processing prior or after the  
25 administration to the cells or to the body.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptides, polypeptides, or analogs thereof, of the present invention. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Any of such salts should have substantially similar activity to the peptides and polypeptides of the invention or their analogs.

The term "derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the N- or C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups.

The proteins of the present invention can be also in the form of active conjugate or complex with a molecule chosen amongst radioactive labels, biotin, fluorescent labels, cytotoxic agents, drug delivery agents. These conjugates or complexes can be generated, using molecules and methods known in the art, for various reasons, for example for allowing the detection of the interaction with CC-chemokines or other proteins (radioactive or fluorescent labels, biotin), for improving therapeutic efficacy (cytotoxic agents), or for improving drug delivery efficacy, using polymers such as polyethylene glycol and other natural or synthetic polymers (Pillai O and Panchagnula R, 2001).

A further object of the invention are CC-chemokine binding peptide mimetics designed on the sequence and/or the structure of the CC-chemokine binding proteins identified in terms of homology to the disclosed sequences of rsChBP-I (SEQ ID NO: 4), avChBP-I (SEQ ID NO: 6), and isChBP-I (SEQ ID NO: 8). Peptide mimetics (also  
5 called peptidomimetics) are molecules in which the nature of peptide or polypeptide has been chemically modified at the level of amino acid side chains, of amino acid chirality, and/or of the peptide backbone. These alterations are intended to provide monomeric variants of the homodimer-forming chemokines having similar or improved properties. Many other modifications providing increased potency, prolonged activity,  
10 easiness of purification, and/or increased half-life are known in the art (WO 02/10195; Villain M et al., 2001). Preferred alternative, "synonymous" groups for amino acids included in peptide mimetics are those defined in Table II. The techniques for the synthesis and the development of peptide mimetics, as well as non-peptide mimetics, are well known in the art (Hruby VJ and Balse PM, 2000; Golebiowski A et al., 2001).  
15 Various methodology for incorporating unnatural amino acids into proteins, using both *in vitro* and *in vivo* translation systems, to probe and/or improve protein structure and function are also disclosed in the literature (Dougherty DA, 2000).

In general, the proteins of the invention may be prepared by any procedure known in the art, including recombinant DNA-related technologies and chemical  
20 synthesis technologies.

A further object of the invention are nucleic acid sequences coding for any of the CC-chemokine binding proteins identified in terms of homology to the disclosed sequences of rsChBP-I (SEQ ID NO: 4), avChBP-I (SEQ ID NO: 6), and isChBP-I (SEQ ID NO: 8), including nucleotide sequences substantially the same (i.e. different

nucleic acid sequences that, by virtue of degeneracy of genetic code, encode for the same protein sequence).

The examples provide the sequence encoding for rsChBP-I, avChBP-I, and isChBP-I, and in particular a novel cDNA, called Clone2 (SEQ ID NO: 3), which  
5 encodes rsChBP-I.

The proteins of the invention can be produced by generating nucleic acid segments encoding them, using common genetic engineering techniques, and cloning in replicable vector of viral or plasmid origin which are used to transform a prokaryotic or eukaryotic host cell, using episomal or non-/homologously integrated vectors, as well  
10 as transformation-, infection-, or transfection-based technologies. These vectors should allow the expression of the recombinant proteins in the prokaryotic or eukaryotic host cell under the control of their own transcriptional initiation/termination regulatory sequences, which are chosen to be constitutively active or inducible in said cell. A cell line substantially enriched in such cells can be then isolated to provide a stable cell line  
15 expressing the protein of interest.

A typical Expression vectors should comprise:

- a) a DNA sequence coding for a CC-chemokine binding proteins identified in terms of homology to the disclosed sequences of rsChBP-I (SEQ ID NO: 4), avChBP-I (SEQ ID NO: 6), and isChBP-I (SEQ ID NO: 8), including  
20 nucleotide sequences substantially the same; and
- b) an expression cassette;

wherein said sequence (a) is operably associated with a tissue specific or a constitutive promoter included in sequence (b).

A process of preparation of a CC-chemokine binding protein of the invention  
25 comprise culturing the transformed host cells and collecting the expressed proteins

Many books and reviews provides teachings on how to clone and produce recombinant proteins using vectors and prokaryotic or eukaryotic host cells, such as some titles in the series "A Practical Approach" published by Oxford University Press ("DNA Cloning 2: Expression Systems", 1995; "DNA Cloning 4: Mammalian Systems", 1996; "Protein Expression", 1999; "Protein Purification Techniques", 2001).

Examples of chemical synthesis technologies for proteins are solid phase synthesis and liquid phase synthesis. As a solid phase synthesis, for example, the amino acid corresponding to the C-terminus of the peptide to be synthesized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the C-terminus to the N-terminus, and one where the amino acids bound to the resin or the protective group of the amino groups of the peptides are released, the peptide chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Cl<sub>2</sub>-Bzl (2,6-dichlorobenzyl) for the amino groups; NO<sub>2</sub> (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino groups; and tBu (t-butyl) for the hydroxyl groups). After synthesis of the desired peptide, it is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or trifluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method.

Totally synthetic proteins of a length comparable to the one of the proteins of the invention are disclosed in the literature (Brown A et al., 1996).

A further object of the invention are purified preparations of proteins, peptide, mimetics, and nucleic acids of the invention.

5 In particular, the purification of synthetic or recombinant proteins defined above can be carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using  
10 monoclonal antibodies or affinity groups, which bind the target protein and are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by heparin or by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or  
15 ionic strength. Alternatively, HPLC (High Performance Liquid Chromatography) can be used. The elution can be carried using a water-acetonitrile-based solvent commonly employed for protein purification.

A further object of the invention is the use of the proteins of the invention as CC-chemokine antagonists, in particular as antagonists said CC-chemokine binding the  
20 chemokine receptor CCR1, such as CCL3 / MIP-1alpha, or a non-mammalian CC-chemokine analogue, such as the ones eventually produced by the tick or by a tick-borne pathogen.

A further object of the invention is the use of the proteins of the invention as medicaments. Given the involvement of CC-chemokines in many human and veterinary  
25 disorders, the CC-chemokine binding proteins of the invention can be used as active

ingredients in pharmaceutical compositions for the treatment or prevention of CC-chemokine related disorders in animals. A non-exhaustive lists of these disorders includes: inflammatory diseases, autoimmune diseases, infections, allergic diseases, cardiovascular diseases, metabolic diseases, gastrointestinal diseases, sepsis, cancers, transplant rejections, or fibrosis.

Non-limitative examples of the autoimmune or inflammatory diseases above indicated are the following: arthritis, rheumatoid arthritis (RA), psoriatic arthritis, osteoarthritis, systemic lupus erythematosus (SLE), systemic sclerosis, scleroderma, polymyositis, glomerulonephritis, fibrosis, fibrosis, allergic or hypersensitivity diseases, dermatitis, asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), Crohn's diseases, ulcerative colitis, multiple sclerosis, septic shock, HIV-infection, transplantation, graft-versus-host disease (GVHD) and atherosclerosis.

Moreover, the proteins of the invention or specific fragments can be used as active ingredients in pharmaceutical compositions for the vaccination of a mammal against parasites, virus, or bacteria.

A pharmaceutical composition containing a protein of the invention as active ingredient can be used for binding a CC-chemokine in vivo, or in blocking binding of a CC-chemokine to a corresponding cell surface receptor in vivo, to produce an immunomodulatory effect, in particular for producing an anti-inflammatory effect.

A pharmaceutical composition containing a protein of the invention as active ingredient, can be used also for binding to CC-chemokine analogue present in virus, bacteria, or parasites to block entry of said virus, bacteria, or parasite into cells.

The compositions above indicated can further comprise an additional immunosuppressant or anti-inflammatory substance.

Pharmaceutical compositions for vaccination of a mammal against a parasite, a virus or a bacteria, can comprise a fragment of the protein of the invention as active ingredient.

The pharmaceutical compositions of the invention may also contain any suitable  
5 pharmaceutically acceptable carriers, biologically compatible vehicles and additives that are suitable for administration to an animal (for example, physiological saline) and eventually comprising auxiliaries (like excipients, stabilizers or diluents) that facilitate the processing of the active compounds into preparations which can be used pharmaceutically. The pharmaceutical compositions may be formulated in any  
10 acceptable way to meet the needs of the mode of administration. For example, the use of biomaterials and other polymers for drug delivery, as well the different techniques and models to validate a specific mode of administration, are disclosed in literature (Luo B and Prestwich GD, 2001; Cleland JL et al., 2001).

"Pharmaceutically acceptable" is meant to encompass any carrier, which does  
15 not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

20 Any accepted mode of administration can be used and determined by those skilled in the art to establish the desired blood levels of the active ingredients. For example, administration may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. Parenteral administration can be by bolus injection or by gradual  
25 perfusion over time. Preparations for parenteral administration include sterile aqueous



or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients known in the art, and can be prepared according to routine methods. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances increasing the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Pharmaceutical compositions include suitable solutions for administration by injection, and contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound together with the excipient. Compositions that can be administered rectally include suppositories.

It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art. The total dose required for each treatment may be administered by multiple doses or in a single dose. The pharmaceutical composition of the present invention may be administered alone or in conjunction with other therapeutics directed to the condition, or directed to other symptoms of the condition. Usually a daily dosage of active ingredient is comprised between 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1 to 40 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage, which is the same, less than, or greater than the initial or previous dose administered to the individual.

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the course and the severity of the disease, leading to the reduction or remission of such pathology. The effective amount will depend on the route of administration and the condition of the patient.

5           The present invention also provides antibodies or an antiserum that is reactive with a tick CC-chemokine binding protein, in particular the ones identified in terms of homology to the disclosed sequences of rsChBP-I (SEQ ID NO: 4), avChBP-I (SEQ ID NO: 6), and isChBP-I (SEQ ID NO: 8), and that can be raised by immunising an animal with these proteins.

10           A further object of the invention are test kit containing any of the compound disclosed in association to the CC-chemokine binding proteins of the invention (including peptide mimetics, nucleic acids, and antibodies). These kits can be used for detecting or measuring a CC-chemokine, CC-chemokine analogue, or CC-chemokine receptor in vitro or in vivo. The kit may comprise a labelled or immobilised reactant,  
15           and/or a compound of the invention that is immobilised on a solid support.

A method of detecting or measuring a CC-chemokine may comprises contacting a sample, possibly containing said CC-chemokine, with a compound of the invention.

An method for detecting a test compound that affects CC-chemokine binding of a protein of the invention may comprise:

- 20           a) bringing into contact a CC-chemokine, said CC-chemokine binding protein and a test compound, under conditions in which in the absence of the test compound being an inhibitor, the CC-chemokine and said CC-chemokine binding protein of the invention interact; and
- b) determining interaction between CC-chemokine and said CC-chemokine  
25           binding protein of the invention.

Further objects of the invention are methods of immunising an animal against a blood-feeding ectoparasite comprising administering to said animal with a compound of the invention, and method for the treatment or prevention of CC-chemokine related diseases, comprising the administration of an effective amount of CC-chemokine binding proteins of the invention.

The therapeutic applications of the polypeptides of the invention and of the related reagents can be evaluated (in terms of safety, pharmacokinetics and efficacy) by the means of the *in vivo* or *in vitro* assays making use of animal cell, tissues and models allowing to detect an inhibition of CC-chemokine receptor binding and/or activation (Methods Mol. Biol. vol. 138, "Chemokines Protocols", edited by Proudfoot A et al., Humana Press Inc., 2000; Methods Enzymol, vol. 287 and 288, Academic Press, 1997). A non-limiting list of assays includes: calcium mobilisation, degranulation, upregulation of pro-inflammatory cytokines, upregulation of proteases, inhibition of cellular recruitment *in vitro* and *in vivo*.

Further characterization of the CC-chemokine binding activities described in the present invention can be obtained by applying various molecular biology technologies recently improved for the study of tick and tick-borne pathogens, such as two-dimensional gel electrophoresis (Madden RD et al., 2002) or RNA interference (Aljamali MN et al., 2003).

Limited information is available on the genome and the transcriptome of haematophagous arthropods, and mostly is associated to ribosomal and mitochondrial sequences, which were studied for determining, on the basis of their conservation, the phylogenetic relationships (Murrell A et al., 2001). Tick genomic data are available only in partial and preliminary formats (Ullmann AJ et al., 2002), but further analysis of the tick genes encoding for CC-chemokine proteins can be performed by using the

genomic DNA, that can be extracted from ixodid ticks by applying specific methods and conditions (Hill CA and Gutierrez, J A 2003), in particular for detecting any significant polymorphism in salivary gland proteins, as already demonstrated (Wang H et al., 1999). The genomic and protein sequences of these organisms is important for understanding their physiology and biology, therefore providing information useful for understanding the role of the proteins of the invention in host, parasite, and parasite-born pathogens relationships (Valenzuela JG, 2002b).

Moreover, the present invention provides novel biological targets for chemotherapeutic chemistries, and novel vaccine therapies for control for tick parasites and tick-borne pathogens.

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions, which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

## EXAMPLES

### **Example 1: biochemical characterization of chemokine-binding activities in the saliva of *Rhipicephalus sanguineus* (dog tick)**

The saliva of the tick *Rhipicephalus sanguineus* has been already used to identify molecules having immunomodulating activities (Matsumoto K et al., J Vet Med Sci 2003; Matsumoto K et al., 2001; Ferreira BR and Silva JS, 1998) but not binding or modulating activities directed specifically to CC-chemokines.

Crude *Rhipicephalus sanguineus* tick saliva was obtained according to the protocol as published (Ferreira BR and Silva JS, 1998). Aliquots of *Rhipicephalus sanguineus* saliva extracts (rsSE) were tested using different assays including, as negative control, Bovine Serum Albumin (BSA) and, as positive control, an *ectromelia* virus protein (called vCCI or p35) binding specifically CC-chemokines (Smith VP and Alcamí A, 2000; Alcamí A, 2003), in order to compare binding specificity and dose-response effects.

In a first assay, different amounts of rsSE and of vCCI were spotted onto nitrocellulose filters in parallel, each of them exposed to a different radiolabeled, recombinant CC-chemokine (CCL / MCP-1, CCL3 / MIP-1 $\alpha$ , and CCL5 / RANTES) or CXC-chemokine (CCL8 / Interleukin 8). While no BSA binding was detected with any radiolabeled chemokine, a CC-chemokine specific binding activity, comparable to the one detected using vCCI, was also detected on filters incubated with any of the CC-chemokines. At the same time, no binding was observed on both rsSE and vCCI spotted filters when incubated with radiolabeled CXCL / Interleukin-8 (Figure 1).

In a second assay, rhSE and vCCI were challenged with specific chemokine / chemokine receptor pairs using the Scintillation Proximity Assay (SPA), a bead-based technology allowing to measure molecular interactions with great precision. In particular, a specific SPA was designed for detecting molecules interfering with the chemokine / chemokine receptor interaction (Alouani S, 2000). Wheat germ agglutinin SPA beads were coated with cell membranes isolated from stably transfected CHO cells expressing a specific chemokine receptor (such as CCR1 or CXCR2) and then incubated with the radiolabeled chemokine alone, in combination with the natural chemokine, or in combination with different amounts of rhSE.

This assay showed that the interaction between CC-chemokines, in particular for the ones binding CCR1 (CCL3 / MIP-1alpha and CCL5 / RANTES) is competed by rhSE in a dose dependent manner (Figure 2). The same assay, when applied for the CXCR2 / CXCL8 pair, confirmed the negative results obtained with the spotted  
5 nitrocellulose filters.

A cross-inhibition SPA experiment was performed by using a CXC-chemokine competitor in presence of a radiolabeled CC-chemokine / chemokine receptor pair and vice versa. The CXC-chemokine (CXCL8 / Interleukin 8) does not interfere with the rhSE-mediated inhibition of the CCR1 / CCR 5 binding of a radiolabeled CC-chemokine  
10 (CCL3 / MIP-1alpha), confirming the specificity of the binding activity in rhSE for CC-chemokines.

Similar CC-chemokine binding activities were also detected with the assays above described in the saliva of *Ambylomma* tick species, indicating that other tick species express CC-chemokine binding activities.

15 Moreover, cross-linking experiments using rhSE and radiolabeled chemokines showed that the cross-linking reagent (bis(sulphosuccinimidyl)suberate or BS3) generates a molecular species having an apparent total molecular weight of approx. 20 kDa when separated in SDS-PAGE. Since radiolabeled CCL3 / MIP-1alpha migrates in SDS-PAGE as 8 kDa protein, rhSE expresses a CC-chemokine binding protein having  
20 a molecular weight in the range of 10-15 kDa.

#### **Example 2: construction and screening of a *Rhipicephalus sanguineus* cDNA library and characterization of rsChBP-I**

The CC-chemokine binding activity identified in rhSE was then identified at the  
25 level of DNA / protein sequence by generating a cDNA library from *Rhipicephalus*

*sanguineus* salivary glands that was then used to produce pools of mammalian cells expressing such cDNAs as proteins secreted in the culture medium.

By comparing the CC-chemokine binding activity detected in culture medium obtained from vCCI expressing cells (or culture medium "spiked" with recombinant vCCI) as a control, these media were then screened using a radiolabeled CC-chemokine (CCL3 / MIP-1alpha), starting from pools of cells and progressively reducing to single cDNA clones.

Human embryonic kidney cells 293 (HEK293 cells; ATCC Cat. No. CRC-1573; maintained in DMEM-F12 Nut Mix, 10% heat-inactivated fetal calf serum, 2 mM L-Glutamine, 100 units/ ml penicillin-streptomycin solution) were chosen to express both vCCI and the cDNA library from *Rhipicephalus sanguineus* salivary glands.

Culture medium from HEK293 cells were obtained from cells grown in complete medium. After three days in culture, the conditioned culture medium was harvested, centrifuged to remove cell debris and the supernatant used in a crosslinking or SPA assay.

The crosslinking experiments were performed on samples transferred to a flat-bottom 96-well plate (Costar). The radiolabeled CC-chemokine (50 µl of 0.23 nM <sup>125</sup>I-CCL3 / MIP-1alpha) was added to each sample, which was then incubated with shaking for 2 hours at room temperature. A 25 µl aliquot from each well was then transferred to another well to containing 0.5 µl of 50 mM BS3 (crosslinking reagent) and further incubated for 2 hours with shaking. After this time 5 µl of 10X sample buffer (0.1 M Tris-HCl pH 8 and 10 mM DTT) were added to each well to stop the crosslinking reaction. The samples were then boiled for 5 minutes and electrophoresed on a 10% Bis-Tris SDS-polyacrylamide gel (Invitrogen NuPAGE, catalog no. NP0301BOX). After electrophoresis the gel was sealed in Saranwrap<sup>TM</sup> and exposed to a K-type storage

phosphor-imaging screen (Biorad) for 8 hours. Imaging screens were scanned at a resolution of 100 um using a Biorad Personal FX phosphoimager

The *Rhipicephalus sanguineus* cDNA library was constructed into pTriplEx2 (BD Biosciences Clontech). Salivary glands were harvested from 100 adult *Rhipicephalus sanguineus* and were immediately stored in ice-cold RNAlater solution (Ambion) until further use. Total RNA was extracted using the TRIzol method (Gibco-BRL) according to the manufacturer's instructions, and the cDNA library was constructed using the SMART cDNA library construction kit (Clontech) and cDNA size-fractionated with a ChromaSpin 400 column (Clontech) according to the manufacturers instructions. The size of the cloned cDNA inserts ranged from about 0.6 kb to 1.5 in 80% of the inserts.

Both DNA sequence encoding for control protein vCCI (Genbank Acc. no. AJ277111; SEQ ID NO: 1) and the cDNA library from *Rhipicephalus sanguineus* salivary glands were subcloned in the pEXP-lib expression plasmid (BD Biosciences Clontech).

The pEXP-Lib vector contains an expression cassette comprising the human cytomegalovirus (CMV) major immediate early promoter/enhancer followed by a multiple cloning site including Sfi IA and Sfi IB sites (two distinct Sfi I sites that differ in their interpalindromic sequences), by an internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA, by the gene encoding puromycin resistance (puromycin-N-acetyl-transferase), and by the polyadenylation signal of the bovine growth hormone. Ribosomes can enter the bicistronic mRNA either at the 5' end to translate the gene of interest in the proper orientation, or at the ECMV IRES to translate the antibiotic resistance marker. When culturing pEXP-Lib Vector transformed cells, the antibiotic exerts selective pressure on the whole expression cassette; thus, a



high dose of antibiotic (10-100 µg/ml of puromycin) select only cells expressing a high level of the gene of interest. This selective pressure also ensures that the expression of the gene of interest will be stable over time in culture.

HEK293 cells were transfected with pEXP-Lib plasmids using a GenePorter2  
5 transfection kit (Gene Therapy Systems) according to the manufacturer's protocol.

The vCCI protein sequence (SWISSPROT Acc No. CAC05575) was detected in the culture medium of HEK cells mixed with a <sup>125</sup>I-labelled CCL3 / MIP-1alpha using BS3 (a cross- linking reagent) either when the medium was "spiked" with recombinant vCCI and when the medium was from vCCI-expressing HEK293. The addition of the  
10 cross-linking reagent generates a protein complex containing the radiolabeled CC-chemokine complex that is separated in SDS-PAGE as a band migrating at ~40-45 kDa. This cross-linking method is very sensible since even complexes in the nanogram weight range can be detected (Figure 3A).

When this method is applied to HEK293 cells transformed with a *Rhipicephalus*  
15 *sanguineus* cDNA library, it was possible to screen, starting from pools of cells, a single clone (Clone2) expressing a CC-chemokine binding protein (identified as rsChBP-I) that forms a complex with radiolabeled CCL3 / MIP-1alpha migrating in SDS-PAGE as a band of approx. 20 kDa (Figure 3B). This band, which is centered in the same weight range of the native CC-chemokine binding activity detected in the tick saliva, appears  
20 as a smear probably due to the presence of isoforms having different levels of glycosilation.

The cDNA encoding for rsChBP-I expressed by Clone2 was sequenced. This 585 bp long cDNA (SEQ ID NO: 3) contains an Open Reading Frame (ORF) encoding for a 111 amino acid (SEQ ID NO: 4), potentially secreted and having no significant  
25 homology with any known CC-chemokine binding protein (Figure 4). Given the

molecular weight, this rsChBP-I sequence should correspond at least one of the CC-chemokine binding activities identified in the tick saliva.

The sequence of rsChBP-I is very similar to a protein sequence encoded by an ORF in a non-characterized 515 bp long cDNA (GenBank Acc. No. BM289643; SEQ ID NO: 5) isolated from salivary glands of *Amblyomma variegatum* (Nene V et al., 2002) and by an ORF in a non-characterized 396 bp long cDNA (GenBank Acc. No. AF483738; SEQ ID NO: 7) isolated from salivary glands of *Ixodes scapularis* (Valenzuela JG et al., 2002). This two additional protein sequences (identified avChBP-I and isChBP-I; Figure 5) contains several conserved cysteines (residues 40, 59, 64, 76, 86, 98, and 99 in rsChBP-I), and are homologous to the screened *Rhipicephalus sanguineus* sequence also in terms of protein length (around 110 amino acids).

The CC-chemokine binding properties of the protein encoded by Clone2 were also tested using the SPA-based approach. As shown for the rsSE (Figure 2), the SPA signal measured in presence of rsChBP-I is inversely proportional to the amount of HEK293-Clone2 culture medium added to the sample, with a dose-dependent inhibition effect on the binding of radiolabeled CCL3 / MIP-1alpha to the SPA beads (Figure 6).

Therefore, it can be concluded that rsChBP-I, avChBP-I, and isChBP-I are component of a novel family of proteins having CC-chemokine binding properties, corresponding to one of the tick protein families of unknown function displaying different conserved cysteine residues.

These proteins may be usefully applied in human medicine as CC-chemokine antagonists, as well as in problems of medical and veterinary public-health importance related to the parasitic effects of ticks, including tick-borne infectious agents. Molecules based on the proteins of the invention and interfering with the function of such proteins,

might disrupt tick life cycle, control ectoparasites and their pathogens, or reduce its ability to transmit disease-causing organisms.

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TABLE I

Amino Acid	Synonymous Group	More Preferred Synonymous Groups
<b>Ser</b>	Gly, Ala, Ser, Thr, Pro	Thr, Ser
<b>Arg</b>	Asn, Lys, Gln, Arg, His	Arg, Lys, His
<b>Leu</b>	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
<b>Pro</b>	Gly, Ala, Ser, Thr, Pro	Pro
<b>Thr</b>	Gly, Ala, Ser, Thr, Pro	Thr, Ser
<b>Ala</b>	Gly, Thr, Pro, Ala, Ser	Gly, Ala
<b>Val</b>	Met, Phe, Ile, Leu, Val	Met, Ile, Val, Leu
<b>Gly</b>	Ala, Thr, Pro, Ser, Gly	Gly, Ala
<b>Ile</b>	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
<b>Phe</b>	Trp, Phe, Tyr	Tyr, Phe
<b>Tyr</b>	Trp, Phe, Tyr	Phe, Tyr
<b>Cys</b>	Ser, Thr, Cys	Cys
<b>His</b>	Asn, Lys, Gln, Arg, His	Arg, Lys, His
<b>Gln</b>	Glu, Asn, Asp, Gln	Asn, Gln
<b>Asn</b>	Glu, Asn, Asp, Gln	Asn, Gln
<b>Lys</b>	Asn, Lys, Gln, Arg, His	Arg, Lys, His
<b>Asp</b>	Glu, Asn, Asp, Gln	Asp, Glu
<b>Glu</b>	Glu, Asn, Asp, Gln	Asp, Glu
<b>Met</b>	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
<b>Trp</b>	Trp, Phe, Tyr	Trp

**TABLE II**

<b>Amino Acid</b>	<b>Synonymous Group</b>
Ser	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Arg	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Leu	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Pro	D-Pro, L-l-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Thr	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Ala	D-Ala, Gly, Aib, B-Ala, Acp, L-Cys, D-Cys
Val	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG
Gly	Ala, D-Ala, Pro, D-Pro, Aib, .beta.-Ala, Acp
Ile	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Phe	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa
Tyr	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Cys	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Gln	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Asn	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Lys	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asp	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Glu	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Met	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val

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## CLAIMS

- 5 1. ~~Proteins having CC-chemokine-binding activity isolated from a tick belonging to~~  
the *Ixodida* suborder.
2. A protein of claim 1, wherein said protein is expressed in the salivary gland  
and/or isolated from the saliva.
3. A protein of claim 1 or 2, wherein the tick belongs to the *Ixodidae* family.
- 10 4. A protein of claim 3, wherein the tick belongs to the *Rhipicephalinae* subfamily.
5. A protein of claim 4, wherein the tick is *Rhipicephalus sanguineus*.
6. A protein of claim 4, wherein said protein has a polypeptide sequence at least  
80% homologous to rsChBP-I (SEQ ID NO: 4).
7. A protein of claim 3, wherein the tick belongs to the *Amblyomminae* subfamily.
- 15 8. A protein of claim 7, wherein the tick is *Amblyomma variegatum*.
9. A protein of claim 7, wherein said protein has a polypeptide sequence at least  
80% homologous to avChBP-I (SEQ ID NO: 6)
10. A protein of claim 3, wherein the tick belongs to the *Ixodinae* subfamily.
11. A protein of claim 10, wherein the tick is *Ixodes scapularis*.
- 20 12. A protein of claim 10, wherein said protein has a polypeptide sequence at least  
80% homologous to isChBP-I (SEQ ID NO: 8).
13. A CC-chemokine binding protein isolated from an organism not belonging to the  
*Ixodida* suborder, wherein said protein has a polypeptide sequence at least 80%  
homologous to rsChBP-I (SEQ ID NO: 4) and has a Cysteine residue in the  
25 positions corresponding to residues 40, 59, 64, 76, 86, 98, and 99 in rsChBP-I.

14. A protein of any of the claims from 1 to 13, wherein said CC-chemokine binds the chemokine receptor CCR1.

---

15. A protein of claim 14, wherein said CC-chemokine is the CCL3 / MIP-1alpha.
16. A protein of any of the claims from 1 to 13, wherein said CC-chemokine is a non-mammalian CC-chemokine analogue.
17. A mature form of the proteins of any of the claims from 1 to 13, wherein said form results from one or more post-translational modifications.
18. A recombinant form of the proteins of any of the claims from 1 to 13.
19. Active mutants, variants, or functional equivalents of a CC-chemokine binding protein of claim 6, 9, 12, or 13, wherein one or more amino acid residues have been added, deleted, or substituted without interfering with the CC-chemokine binding activity.
20. An active mutant of claim 19, wherein said active mutant comprises an amino acid sequence belonging to a protein sequence other than the corresponding CC-chemokine binding protein.
21. An active mutant of claim 20, wherein the amino acid sequence belonging to a protein sequence other than the corresponding CC-chemokine binding protein is an amino acid sequence belonging to one or more of these protein sequences: extracellular domains of membrane-bound protein, immunoglobulin constant region, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins.
22. An active mutant of claim 21, wherein the sequence alterations reduce the immunogenicity of said CC-chemokine binding protein when administered to a mammal.

23. A fragment of a CC-chemokine binding protein of claim 6, 9, 12, or 13 having an immunizing activity when administered to a mammal.
- 
24. A protein of any of the claims from 1 to 23, wherein said protein is in the form of an active fraction, precursor, salt, or derivative.
- 5 25. A protein of any of the claims from 1 to 23, wherein said protein is in the form of active conjugate or complex with a molecule chosen amongst radioactive labels, biotin, fluorescent labels, cytotoxic agents, drug delivery agents.
26. CC-chemokine binding peptide mimetics designed on the sequence and/or the structure of the proteins of claim 6, 9, 12, or 13.
- 10 27. Nucleic acid sequences comprising the nucleic acid sequence coding for any of the polypeptide sequences of claim 6, 9, 12, 13, or 19, including nucleotide sequences substantially the same.
28. The nucleic acid sequence of claim 27 wherein the polypeptide sequence is rsChBP-I (SEQ ID NO: 4)
- 15 29. The nucleic acid sequence of claim 28 wherein the nucleic acid sequence is Clone2 (SEQ ID NO: 3)
30. Expression vectors comprising:
- a) a DNA sequence coding for a polypeptide sequence of claim 6, 9, 12, 13, or 19, including nucleotide sequences substantially the same; and
- 20 b) an expression cassette;
- wherein said sequence (a) is operably associated with a tissue specific or a constitutive promoter included in sequence (b).
31. Host cells transformed with vectors of claim 30.

32. Process of preparation of a CC-chemokine binding protein of claim 6, 9, 12, 13 or 19, comprising culturing the transformed cells of claim 31 and collecting the expressed proteins.
- 
33. Purified preparations of compounds of any of the claims from 1 to 27.
- 5 34. Use of the compounds of any of the claims from 1 to 22 as CC-chemokine antagonists.
35. The use of claim 34, wherein said CC-chemokine binds the chemokine receptor CCR1.
36. The use of claim 35, wherein said CC-chemokine is the CCL3 / MIP-1alpha.
- 10 37. The use of claim 34, wherein said CC -chemokine is a non-mammalian CC-chemokine analogue.
38. Use of the CC-chemokine binding proteins of any of the claims from 1 to 27 as medicaments.
- 15 39. Use of CC-chemokine binding proteins of any of the claims from 1 to 22 as active ingredients in pharmaceutical compositions for the treatment or prevention of CC-chemokine related disorders in animals.
40. The use of claim 39 wherein the disorder is an inflammatory disease, an autoimmune disease, an infection, an allergic disease, a cardiovascular disease, a metabolic disease, a gastrointestinal disease, sepsis, cancer, transplant rejection, or fibrosis.
- 20 41. Use of the protein of any of the claims from 1 to 23 as active ingredients in pharmaceutical compositions for the vaccination of a mammal against parasites, virus, or bacteria.

42. A pharmaceutical composition containing a compound of any of the claims from 1 to 22 as active ingredient, for use in binding a CC-chemokine in vivo, or in blocking binding of a CC-chemokine to a corresponding cell surface receptor in vivo, to produce an immunomodulatory effect.
- 5 43. A composition according to claim 42, for use in producing an anti-inflammatory effect.
44. A pharmaceutical composition containing a compound of any of the claims from 1 to 22 as active ingredient, for use in binding to a CC-chemokine analogue present in a virus, a bacteria, or parasite to block entry of said virus, bacteria, or parasite into cells.
- 10 45. A composition according to any one of the claims from 42 to 44, wherein said composition further comprises an additional immunosuppressant or anti-inflammatory substance.
46. Pharmaceutical compositions for vaccination of a mammal against a parasite, a virus or a bacteria, comprising a fragment of claim 23 as active ingredient.
- 15 47. An antibody or an antiserum that is reactive with a tick CC-chemokine binding protein.
48. An antibody or antiserum of claim 47, wherein said protein is a protein of claim 6, 9, or 12.
- 20 49. A method of production of an antibody or an antiserum of either claim 47 or 48, comprising immunising an animal with a compound of claim 23.
50. A test kit containing a compound of any of the claims from 1 to 27, or of claim 47, for detecting or measuring a CC-chemokine, CC-chemokine analogue, or CC-chemokine receptor in vitro or in vivo.

51. A test kit of claim 50, further comprising a labelled or immobilised reactant.
52. A test kit of claim 50, wherein the compound of any of the claims from 1 to 27, or of claim 47, is immobilised on a solid support.
- 
53. A method of detecting or measuring a CC-chemokine, wherein said method  
5 comprises contacting a sample, possibly containing said CC-chemokine, with a compound of any of the claims from 1 to 27, or of claim 47.
54. A method for detecting a test compound that affects CC-chemokine binding of a protein of any of the claims from 1 to 13, wherein said method comprises
- 10 a) bringing into contact a CC-chemokine, said CC-chemokine binding protein and a test compound, under conditions in which in the absence of the test compound being an inhibitor, the CC-chemokine and said CC-chemokine binding protein interact; and
- b) determining interaction between CC-chemokine and said CC-chemokine binding protein.
- 15 55. Method of immunising an animal against a blood-feeding ectoparasite comprising administering to said animal with a compound of claim 23.
56. Method for the treatment or prevention of CC-chemokine related diseases, comprising the administration of an effective amount of CC-chemokine binding proteins of any of the claims from 1 to 22.

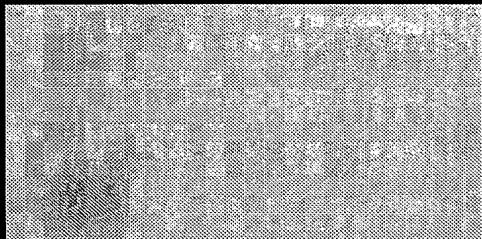
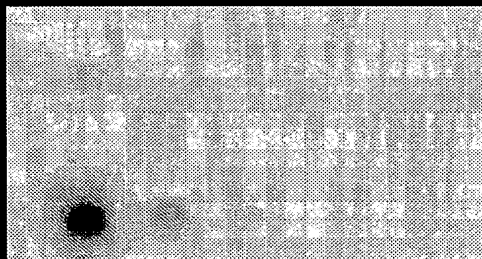
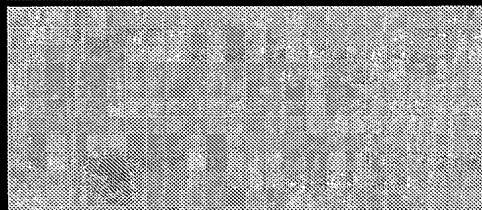
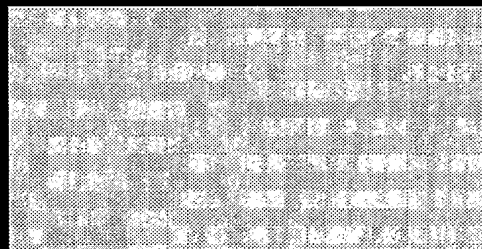
# **ABSTRACT**

CC-chemokine binding activities have been identified in the saliva of *Rhipicephalus sanguineus* (dog tick). In particular, rsChBP-I, a novel protein isolated from a *Rhipicephalus sanguineus* cDNA library and expressed in mammalian cells, competes with a CC-chemokine receptor for CC-chemokine binding. This protein belongs to a new class of tick proteins that can be used therapeutically as antagonists of natural CC-chemokines in mammalian organisms, as well as targets for vaccination and the control of ticks and of tick-borne-pathogens.





**Figure 1**

Spotted Filter					Radiolabeled Chemokine
2	0.2	microliters rhSE			
100	10	1	0.1	µg vCCI	
					CCL2 / MCP-1
					CCL3 / MIP-1alpha
					CCL5 / RANTES
					CXCL8 / Interleukin-8

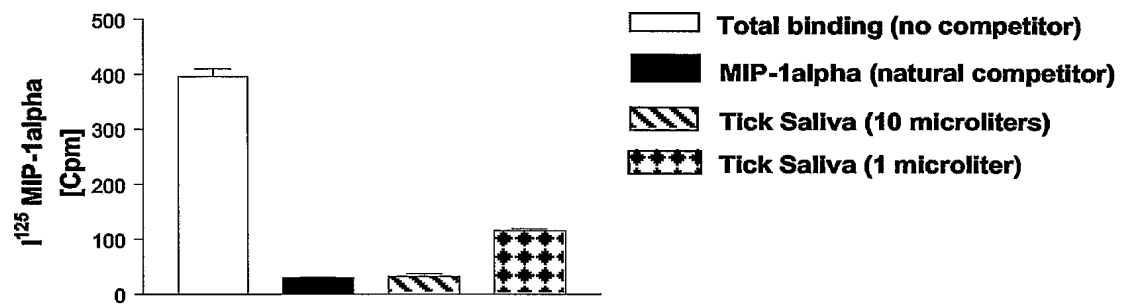
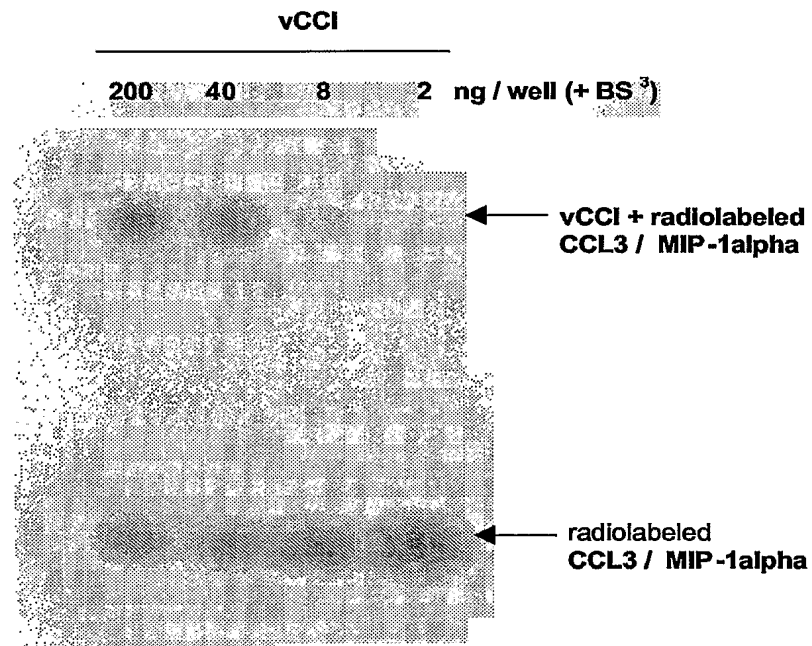
**Figure 2**

Figure 3

A)



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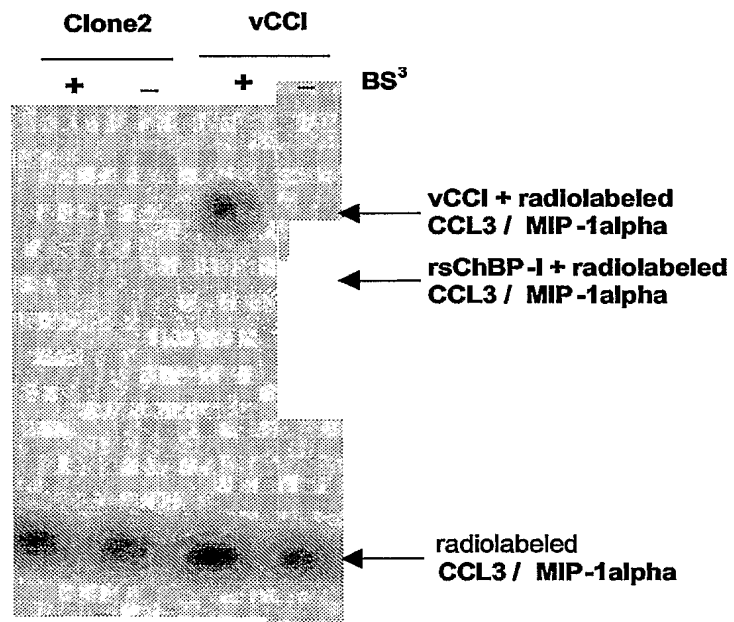


Figure 4

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**Figure 5**

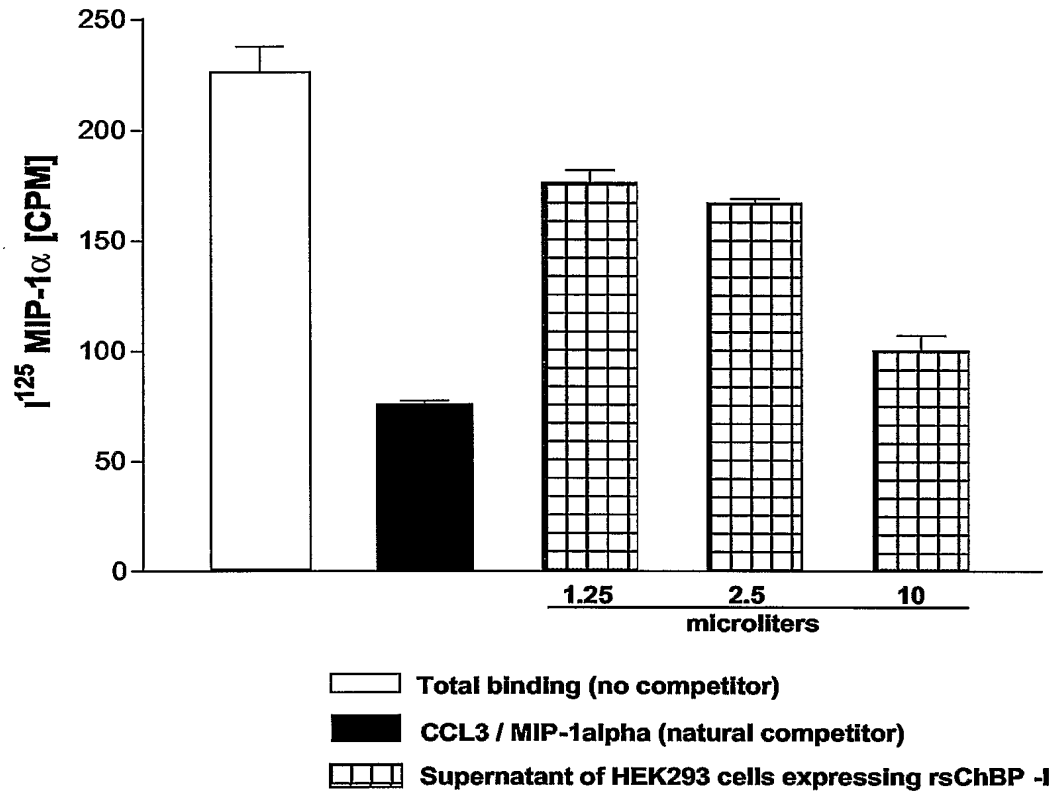
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Figure 6



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